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UVB exposure of a humanized skin model reveals unexpected dynamic of keratinocyte proliferation and Wnt inhibitor balancing

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Abstract

We developed human dermo-epidermal skin substitutes which are presently applied in phase I and II clinical trials. Here we used these very same skin equivalents containing melanocytes, named MelSkin, as an experimental skin model. We investigated the effects of UVB irradiation on the skin grafts transplanted on immune-compromised rats. The irradiation induces a strong wound healing response going along with massive proliferation of basal keratinocytes, basically quiescent under non-irradiated, homeostatic conditions. As a consequence of UVB irradiation, the initially clearly defined basal keratinocyte (mono)layer expands into about three layers of keratinocytes, all of which still express the basal keratinocyte marker Keratin15. In contrast, epidermal melanocytes remain quiescent under these circumstances. Moreover, the Wnt inhibitors Dickkopf 3 and Wif1 are downregulated upon UVB irradiation in basal keratinocytes, whereas melanocytes continue to express Wnt inhibitors. These findings suggest that there is 1) a suprabasal population, proliferating in the homeostatic state, hence maintaining the integrity of the epidermis, and 2) a basal, usually quiescent keratinocyte population that is induced to massively proliferate upon irradiation. Importantly, the finding that MelSkin responds in a physiological fashion to UVB is of paramount importance in light of the planned clinical application.

Keywords: Tissue engineering, human pigmented skin substitute, keratinocytes, melanocytes, WIF1, DKK3, K15, UVB

1. Introduction

Here we describe the first series of experiments investigating the effects of UVB irradiation on a novel type of *pigmented* bio-engineered dermo-epidermal skin substitutes of human origin, termed MelSkin. This new generation of skin equivalents, was investigated *in*

vivo employing immuno-incompetent rats (Biedermann et al. 2015b; Böttcher-Haberzeth et al. 2014; Böttcher-Haberzeth et al. 2013). Of note, basically identical but *non-pigmented* skin substitutes, are currently being tested in phase I and II clinical studies with highly encouraging results. Hence, the experimental setting used here is methodologically meaningful, particularly with regard to future clinical application of MelSkin.

In general, only few studies investigated skin color, melanocyte presence and melanin content in dermo-epidermal skin substitutes containing melanocytes, but describe no UV light stimulation (Swope et al. 2006; Swope et al. 1997). Further, Yoshida et al. described UV irradiation of an *in situ* formed skin, after mixing and pooling of melanocytes, keratinocytes and fibroblasts into a transplantation chamber was performed (Yoshida et al. 2007). In addition, several other studies described the use of pure epidermal substitutes, not dermo-epidermal skin substitutes, containing only keratinocytes and melanocytes for *in vitro* investigations including UV light stimulation, referring as example to melanosome transfer via PAR-2, the effect of DKK1 onto hypopigmentation, or hyperpigmentation caused by endothelin-1 and stem cell factor (Seiberg et al. 2000; Wakabayashi et al. 2013; Yamaguchi et al. 2008).

As in MelSkin the melanocyte compartment is biologically fully functional (Biedermann et al. 2015b; Böttcher-Haberzeth et al. 2014; Böttcher-Haberzeth et al. 2013), we determined how MelSkin would compensate UV irradiation, in terms of skin pigmentation, cell proliferation and epidermal skin homeostasis. UVB irradiation can induce skin tanning as a protective mechanism to DNA damage. It is know that it induces expression of the wound healing marker Keratin 16 (K16) in epidermal keratinocytes (Sano et al. 2009; Chen et al. 2014). Moreover, UVB irradiation induces keratinocyte proliferation in the human epidermis (Lee et al. 2002; Sano et al. 2009; van der Vleuten et al. 1996).

It was shown that after UV exposure epidermal keratinocytes release several factors, such as keratinocyte growth factor, stem cell factor, alpha-MSH, and endothelin-1 that modulate melanocytes (Choi et al. 2010). These paracrine factors, but also autocrine factors, regulate melanocyte behavior after UV irradiation. Therefore, transcription factors, such as MITF and SOX9, and several melanosomal enzymes, such as tyrosinase (TYR), tyrosinase-related protein 2 (DCT), or tyrosinase-related protein 1 (TYRP1), are activated and expressed in melanocytes (López et al. 2015). This eventually increases the production of melanin by melanocytes and leads to tanning of the skin after UV irradiation (Choi et al. 2010).

In mouse skin, UVB irradiation activates melanocyte stem cells residing in the bulge of the hair follicle. As a consequence these cells leave the niche and migrate into the mouse epidermis, giving rise to differentiated, pigment-producing melanocytes (Chou et al. 2013). This developmental transition of melanocyte stem cells to functional melanocytes is triggered by Wnt7a produced by keratinocytes after the UVB irradiation (Yamada et al. 2013). As human skin differs quite significantly from mouse skin - humans have no fur and the melanocyte compartment can be maintained in the complete absence of hair follicles - this transition may not be as relevant.

Using relatively complex dermo-epidermal skin substitutes containing human melanocytes, keratinocytes and fibroblasts, we set out to investigate the effects of UVB stimulation on the expression of basal layer cytokeratins, such Keratin 15 and 19, the wound healing marker K16, the proliferation marker Ki67 in both basal and suprabasal keratinocytes, as well as in melanocytes, and the expression of the Wnt inhibitors Dickkopf 3 and Wif1.

One of the most immediate and obvious effects of UVB irradiation was the massive proliferation of basal keratinocytes. The initially clearly defined Keratin 15 positive basal mono-layer was induced to expand into about three layers of keratinocytes. Surprisingly, all

three keratinocyte layers were Keratin 15 positive, which may be interpreted as a thickening of the stratum basale. Furthermore, UVB irradiation caused a strong wound healing response in all suprabasal keratinocyte layers. Notably, melanocytes did not proliferate and remained quiescent after UVB stimulation. Accordingly, the Wnt inhibitors Dickkopf 3 and Wif1 became downregulated upon UVB irradiation in basal keratinocytes, whereas melanocytes continued to express them. Four to five weeks after UVB irradiation, all described effects disappeared and values reverted to normal. Based on these data and conclusions we would like to point out that MelSkin not only represents a new generation of pigmented bio-engineered skin substitute for clinical application, but also provides a valuable model for experimental and screening purposes.

2. Materials and Methods

Human skin biopsies

Human skin samples were taken from the outer skin layer of foreskins obtained after surgical intervention of patients ranging in age between one and 16 years and after receiving written informed consent by parents or patients. The study was conducted according to the Declaration of Helsinki and with permission of the Ethic Commission of the Canton of Zurich.

Isolation and cell culturing of human keratinocytes, fibroblasts and melanocytes

Epidermal keratinocytes and dermal fibroblasts were isolated from 4 different skin samples (Fitzpatrick skin type II) and cultured as described in Pontiggia et al. 2009 (Pontiggia et al. 2009). Specifications on the protocol of melanocyte isolation and culturing were previously published in (Böttcher-Haberzeth et al. 2013).

Tissue engineering of MelSkin

We prepared 3 MelSkin specimen from each of the 4 different foreskin samples as described previously (Böttcher-Haberzeth et al. 2013; Pontiggia et al. 2009). Briefly, hydrogels functioning as dermal equivalents were prepared using 6-well cell culture inserts with 3.0 µm pore-size membranes (BD Falcon, Switzerland). Rat collagen type-I (BD Biosciences, Allschwil, Switzerland) was mixed with 1,5 x 10^5 human dermal fibroblasts, poured into the wells and polymerized to hydrogels after 30 minutes at 37°C. The hydrogels were compressed using weights one day after fabrication and were cultured for 5 more days in Dulbecco's Modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 5µg/ ml gentamycin (all from Invitrogen, Switzerland). Subsequently 8 x 10^5 keratinocytes and 1,6 x 10^5 melanocytes (5:1 ratio) of the same donor were mixed and seeded onto the fibroblast containing hydrogels. Engineered MelSkin was kept for 24 hours in pure keratinocyte serum free medium (SFM, Invitrogen, Switzerland) and cultured for 5 more days in a 5:1 mix of keratinocyte SFM and melanocyte growth medium, containing Phorbol Myristate Acetate (PromoCell, Heidelberg, Germany). Hydrogels were tested with fluorescein diacetate (FDA)-staining for cell residence prior to transplantation.

Transplantation of MelSkin

The surgical protocol was approved by the Committee for Experimental Animal Research of the University of Zurich (permission number 115/2012) and performed as published in Biedermann et al. (Biedermann et al. 2010). The 12 MelSkin substitutes (3 MelSkin specimen from each of 4 different skin samples) were transplanted onto full-thickness skin defects created surgically on the back of 12 immuno-incompetent female nude (Crl:NIH-Foxn1rnu) rats, 8-10 weeks old (Charles River, Germany).

Isoflurane anesthesia and analgesic treatment was performed as described before (Böttcher-Haberzeth et al. 2013; Pontiggia et al. 2009). To prevent overgrowth of the transplanted human skin by surrounding rat skin, steel rings (2,6 cm diameter) were sutured on the back of the rats, using non-absorbable polyester sutures (Ethibond®, Ethicon, USA). MelSkin substitutes were placed inside the rings and fixed with stiches on the rat muscle fascia. Wound dressing consisted of a primary covering with silicon foil (Silon-SES, BMS, USA) and on top paraffin gauze (Jelonet, Smith&Nephew, England), polyurethane foam dressing (Ligasano, Ligamed, Austria) and tape (Leukoplast® hospital, BSN medical GmbH, Germany). Dressing changes and photographic documentation were performed weekly.

Exposure of grafted MelSkin to UVB and color measurement

4 weeks after transplantation, a single dose of UVB irradiation with an intensity of 250mJ/cm^2 was applied on grafted MelSkin. The employed device (Excimer System, Alma Lasers) is emitting monochromatic narrowband UVB light with a wavelength of 308nm. Grafted MelSkin were then kept another 4 to 5 weeks on the rats. Chromameter measurement and excision of 5mm punch biopsies were performed at wound dressing changes.

Punch biopsies were embedded in OCT compound (Sakura Finetec, Staufen, Germany) and processed for cryo-sections. A chromameter (CR-400, Konica Minolta, Japan), working on the principle of light spectroscopy, was employed to detect alterations in grafted skin color before and after UVB exposure. The measured *L-value* for lightness ranges from absolute black (0) to absolute white (+100). Each sample on the 12 animals was measured at each time point 5 times and the average of all the L-values for this time point was calculated. Due to collection of punch biopsies, MelSkin substitute area was not large enough to perform chromameter measurements later than 4 weeks after UVB exposure.

Histological and immunofluorescence staining

The Fontana Masson method (Böttcher-Haberzeth et al. 2014) was employed for melanin staining. For immunofluorescence stainings for each of the 12 samples at the different time points (without UVB, UVB+1 week, UVB+3 weeks, UVB+4 weeks, UVB+5 weeks) a biopsy was taken. For each biopsy five sections were used to perform each of the displayed stainings. A representative field of the stainings is always shown in the figures as example.

Cryo-sections and cells (from the 4 skin samples) in culture were fixed for 30 minutes at 37 °C, permeabilized with Triton 1% solution for 8 minutes and blocked in 3% BSA-Buffer (Sigma, Buchs, Switzerland) for 10 minutes. Slides were incubated with primary antibody in blocking solution either over night at 4 °C or for one hour at room temperature. After washing three times with PBS, the secondary antibody was added for 40 minutes. Finally slides or cells were incubated for 5 minutes with Hoechst staining (PBS containing 1µg/ ml Hoechst 33342, Sigma, Buchs, Switzerland). The staining was completed with another three washings in PBS and adding Dako mounting solution (Dako, Baar, Switzerland).

Antibodies

The following primary antibodies were used for immunofluorescence: HMB45 (1:50), K10 (clone DE-K10, 1:100), K19 (clone RCK108, 1:100), and K16 from Dako (Switzerland AG, Baar, Switzerland); Integrin alpha6 (clone 4F10, 1:100), K15 (clone LHK15, 1:100) from Chemicon (Milepore AG, switzerland); Ki-67 (clone B56, 1:100) from Abcam (Cambridge, UK); Desmoglein 3 (clone 5G11, 1:100) from Invitrogen (Basel, Switzerland); DKK3 (1:100) from Santa Cruz (Labforce AG, Nunningen, Switzerland); WIF1 (1:100) from R&D (R&D Systems Inc., Minneapolis, USA). As secondary antibodies FITC- and TRITC-

conjugated polyclonal goat anti-mouse or anti-rabbit F(ab')2 fragments (Dako, Baar, Switzerland) were used. In some cases, pre-labeling of primary antibodies for double immunofluorescence, was performed with Alexa 555-conjugated polyclonal goat F(ab')2 fragments, according manufacturer instructions (Zenon Mouse IgG Labeling Kit; Molecular Probes, Invitrogen, Basel, Switzerland).

Fluorescence microscopy

Fluorescence microscopy was performed using a DXM1200F digital camera connected to a Nikon Eclipse TE2000-U inverted microscope, containing Hoechst 33342, FITC, and TRITC filter sets (Nikon, AG, Switzerland; Software: Nikon ACT-1 vers. 2.70). Photoshop 11.0 (Adobe Systems, Germany) was used for image processing.

Western blot analysis

Foreskin epidermis and cultured keratinocytes were both treated equally according to the following protocol. The material was washed twice with cold PBS and lysed at 41°C in RIPA lysis buffer containing 20mM Tris-HCl, pH 7.5, 150mM NaCl, 5mM EDTA, 1% (v/v) Triton X-100, 1mM Na3VO4, protease inhibitor cocktail (Roche, Basel, Switzerland), and phosphatase inhibitor cocktail 1 (Sigma-Aldrich). Proteins were then denatured at 95°C for 5 minutes and separated by a 12% SDS-Polyacrylamide gel electrophoresis under reducing conditions and transferred onto nitrocellulose membranes (Invitrogen). After blocking for 1 hour at room temperature (RT) with 2% low-fat skim milk, membranes were probed with DKK3 antibody (1:500) (clone C-19, Santa Cruz (Labforce AG, Nunningen, Switzerland)) and GAPDH (1:500) (clone 0411, Santa Cruz) in 5% BSA at 4° C overnight. Washing steps with buffer (PBS plus 0.3% Tween 20) were performed 3 times for 10 minutes each.

Horseradish peroxidase–conjugated secondary antibodies (Abcam, Cambridge, UK) were incubated for 1 h at room temperature. Three times washing steps were performed and bound antibodies were detected by chemiluminescence (ECL, GE Healthcare, Buckinghamshire, UK).

Statistical analysis

Keratinocytes (Hoechst positive, HMB45-negative) and melanocytes (Hoechst positive, HMB45-positive) were counted in high-power fields in five representative sections of each skin sample. Statistical analysis containing mean value, standard deviation and unpaired students t-test were performed with Microsoft Excel. $p < 0,05$ was considered significant.

3. Results

UVB exposure leads to reversible tanning in MelSkin *in vivo*

MelSkin grafts when transplanted onto the backs of nu/nu rats showed restored pigmentation matching to the original color of the donor skin 4 weeks after transplantation (Figure 1a, see also Böttcher-Haberzeth et al., 2013). When treated with a single dose of 250mJ/cm^2 UVB irradiation, tanning was observed in all MelSkin substitutes 1 week after irradiation (Figure 1b). Chromameter measurements were employed to objectively determine skin color (Figure 1d). The L-value correlating to perceived lightness and ranging from absolute black (0) to absolute white (+100), was significantly reduced 1 week after UVB exposure (reduction of the mean L-value from 57,93 to 49,64, $p<0,001$), indicative for a darker skin color. Tanning was still observed 4 weeks after irradiation (Figure 1c), although a less tanned skin was clearly indicated by the L-values (Figure 1d). Punch biopsies of MelSkin were collected to perform Fontana-Masson stainings at defined time points. Before UVB

treatment, melanin was mainly observed in the keratinocytes of the basal layer and first suprabasal layers (Figure 1e). Two days after UVB exposure, melanin caps covering the basal and first suprabasal keratinocytes in non-irradiated control skin were extended to the keratinocytes also in the most upper suprabasal layers (Figure 1f).

A wound situation is transiently induced in MelSkin after UVB irradiation

Our previous studies have revealed that the wound healing marker Keratin 16 (K16) was strongly expressed in epidermal keratinocytes early after transplanting MelSkin. K16 expression disappeared 3 - 4 weeks thereafter when skin homeostasis was reestablished (Biedermann et al. 2010; Hartmann-Fritsch et al. 2013). We, therefore, used the K16 expression status as an indicator for tissue homeostasis in transplanted MelSkin. As soon as K16 was no longer detectable in the epidermis, after 4 weeks, a single dose of UVB irradiation with an intensity of 250mJ/cm^2 was applied on MelSkin grafts. K16 expression was induced in all suprabasal layers 1 week after UVB irradiation, indicating a wound condition (Figure 1h). MelSkin recovered from this wound condition 3 - 4 weeks after irradiation displaying again a K16 negative epidermis (Figure 1i).

MelSkin grafts showed a typical expression pattern of Desmoglein 3 in the spinous layers of the epidermis 4 weeks after transplantation (Figure 1j) (Biedermann et al., 2010). In accordance to the transient expression of K16, we observed increased expression of Desmoglein 3 also in granular layers of the epidermis (Figure 1k) one week after UV irradiation. Again, Desmoglein 3 expression 3 - 4 weeks after irradiation decreased to the initial homeostatic expression, i.e. to levels seen before irradiation (Figure 1l).

A subpopulation of K19 and K15 positive basal keratinocytes is activated after UVB exposure of MelSkin

We reported previously about a subpopulation of K15/K19 positive basal keratinocytes, which was seen in dermo-epidermal skin grafts, early after transplantation. In all cases K19 expressing keratinocytes became restricted to the stratum basale before they disappeared after skin homeostasis was established (Pontiggia et al. 2009; Biedermann et al. 2010; Hartmann-Fritsch et al. 2013).

In MelSkin, K15 was homogeneously expressed in the basal layer at about 4 weeks after transplantation (Figure 2a, a''). In contrast only few single K19 positive keratinocytes were found scattered in the stratum basale (Figure 2a, a'). It came as a surprise that UVB exposure caused K15 positive keratinocytes, usually clearly restricted to the stratum basale, to expand into several (3 to 4) layers (Figure2b, b''). The same was true for K19 expressing keratinocytes. Three weeks after UVB irradiation the number of both K15 and K19 positive keratinocytes was largely reduced and both types of keratinocytes were again restricted to the stratum basale. K19 positive keratinocytes were less abundant than K15 expressing cells (Figure 2c).

UVB irradiation induces massive proliferation of basal keratinocytes

Immunofluorescence staining for Ki67 of MelSkin transplant biopsies was performed to detect proliferating keratinocytes at different time points. We found that Keratin 10 (K10) expression was restricted to suprabasal cells and therefore used it to distinguish suprabasal layers (K10 positive) from the basal layer (K10 negative) (Figure 3a,b,c).

As demonstrated in Figure 3a there were only few Ki67 positive keratinocytes in MelSkin 4 weeks after transplantation. Interestingly about 70% of these cells were typically found in the first (K10 positive) suprabasal layers (Figure 3a and 3e). To gain quantitative data on the location of Ki67 positive keratinocytes, these cells were counted per 100 basal

cells and classified as basal (K10 negative) or suprabasal (K10 positive) cells. The resulting diagram (Figure 3d) shows a very low cell proliferation rate in the basal layer and some proliferating keratinocytes in the first 2-3 suprabasal layers before UVB treatment. We found a massive increase of Ki67-positive basal keratinocytes already 2 days after UVB irradiation. This massive induction of basal keratinocyte proliferation explains the expanded stratum basale, which was "blown up" to about 3 layers (Figure 2b) after UVB irradiation. Notably, suprabasal keratinocytes did not yet show increased proliferation at this time after irradiation (Figure 3d). One week after UVB irradiation the proliferation rate of basal keratinocytes was still high. In contrast, there was now a significant increase of suprabasal keratinocyte proliferation (Figure 3b, d). Two weeks after UVB treatment, the proliferation rate in both compartments decreased significantly remaining at an intermediate level for about two weeks (Figure 3d). At about 5 weeks after the UVB treatment the proliferation rate of suprabasal keratinocytes dropped to its initial level, while proliferation of basal keratinocytes was still somewhat elevated (Figure 3d).

To confirm the spatial distribution of Ki67 expressing epidermal keratinocytes by an additional approach, we determined their position in relation to the basement membrane. The keratinocytes contacting the basement membrane were made visible by Integrin alpha-6 staining (Figure 3e, f, g). Before UVB irradiation and 5 weeks thereafter, Ki67 expressing keratinocytes were typically located in suprabasal layers. Rarely Ki67 expressing keratinocytes were found in contact with the basement membrane, hence in the basal layer (Figure 3e,g). One week after UVB treatment, a massive increase of Ki67 positive keratinocytes was observed. Again these Ki67-positive keratinocytes were distributed in several layers. Many of these keratinocytes expressed the alpha unit of the Lam5 receptor Integrin α6β4 (Figure 3f).

To determine the number of proliferating melanocytes in MelSkin after UVB irradiation, a double staining using the antibodies Ki67 and Human Melanoma Black 45 (HMB45), a marker for pre-melanosomes in melanocytes, was performed. With very few exceptions, no Ki67/HMB45 double expressing melanocytes were detected one week after UVB irradiation (Figure 3h). As indicated in Figure 3i the numbers of melanocytes remained constant within the period of 5 weeks after UVB irradiation. From these findings we conclude that both basal and suprabasal keratinocyte are subsequently induced to enter the cell cycle (and proliferate) by UVB exposure, whereas melanocyte numbers remain constant under these circumstances.

UVB-induced keratinocyte proliferation goes along with suppression of Wnt inhibitor expression in the stratum basale of MelSkin

We show here for the first time in human epidermis the typical subcellular DKK3 protein expression with vesicle-like spots around the nucleus, as it has been described for other human cell types and murine epidermis (Zhang K et al. 2010; Lim et al. 2013). We found this characteristic expression pattern in few keratinocytes in 2D culture, notably negative for Ki67 (Figure 4a), in human foreskin (Figure 4b), as well as in MelSkin (Figure 4d-f). In MelSkin four weeks after transplantation DKK3 was detected in lower suprabasal layers and predominantly, also in the basal layer (Figure 4d). One week after UVB exposure DKK3 expression was suppressed in the vast majority of basal cells. Notably, Ki67 positive cells were negative for DKK3 (Figure 4e). DKK3 expression in MelSkin 5 weeks after UVB exposure returned to its initial distribution pattern, with DKK3 present particularly in basal cells (Figure 4f). When employing western blot analysis, keratinocytes from normal human foreskin epidermis presented with clearly higher amount of DKK3 protein than proliferating keratinocytes in culture (Figure 4c).

In line with these findings was the expression of the Wnt inhibitor WIF1 in 2D-culture and in MelSkin. Keratinocytes in culture expressing WIF1 did not express Ki67 (Figure 5 a). *In vivo*, WIF1 was present in most epidermal and dermal cells of human foreskin (data not shown). This was also observed in MelSkin 4 weeks after transplantation (Figure 5b). Notably, all basal keratinocytes highly expressed WIF1 before UVB exposure. The few single Ki67 positive keratinocytes were WIF1 negative. One week after UVB treatment overall WIF1 expression in MelSkin decreased. In particular, most basal cells lost WIF1 expression. However, few basal cells continued to express WIF1 and were Ki67 negative (Figure 5c).

To analyze whether melanocytes in the basal layer express WIF1, we employed WIF1/HMB45 double staining of MelSkin (Figure 5d-f). Interestingly, melanocytes showed a strong expression of WIF1 before and after UVB irradiation. Notably, after UVB treatment, WIF1 positive cells in the basal layer, were not only melanocytes. A few basal keratinocytes also continued to express WIF1 suggesting that these keratinocytes remained in a quiescent, non-proliferative state (Figure 5e,f).

4. Discussion

We have employed a novel type of *pigmented* bio-engineered dermo-epidermal skin substitutes of human origin (MelSkin) to investigate the effects of UVB irradiation *in vivo*, i.e. in a model of immuno-incompetent rats. Of note, basically identical, but *non-pigmented,* skin substitutes are currently being tested in phase I and II clinical studies with encouraging preliminary results after they have been extensively and successfully tested on immune-compromised rats. Hence, the experimental setting used here appears methodologically meaningful, particularly with regard to future clinical application of MelSkin.

We recently demonstrated for MelSkin the regular distribution of melanocytes in the basal layer with the protrusion of dendrites into lower and upper keratinocyte layers and the

deposition of supranuclear melanin caps in the keratinocytes (Biedermann et al. 2015b). Moreover, the original color of the donor skin was restored after transplantation (Biedermann et al. 2015b; Böttcher-Haberzeth et al. 2013). Distinct MelSkin substitutes differing in color (light or dark) showed physiological melanocyte distribution and function even 15 weeks after transplantation (Biedermann et al. 2015a; Böttcher-Haberzeth et al. 2014). We show here that MelSkin can adapt its melanin content as a reaction to UVB exposure, namely by an increased production of melanin and its transfer to the upper layers of epidermal keratinocytes. Eventually, this resulted in tanning of MelSkin.

The wound healing marker K16 became highly expressed in engineered *non-pigmented* skin substitutes early after transplantation. Its expression was completely shut off 3 to 4 weeks after transplantation. This correlated with the establishment of epidermal homeostasis (Biedermann et al. 2010; Hartmann-Fritsch et al. 2013). Accordingly, we saw K16 highly expressed in MelSkin one week after UVB irradiation, and disappearing 3 to 4 weeks after irradiation. Taken together these findings reveal a highly physiological response of MelSkin to UVB irradiation, underlining its potential for both clinical applications as well as experimental purposes.

K19 expression is temporarily reactivated upon UVB irradiation

Our previous studies (Pontiggia et al. 2009; Biedermann et al. 2010; Hartmann-Fritsch et al. 2013) have revealed, that the transient expression of K19 in suprabasal cell layers of the epidermis early after transplantation corresponds with a still non-homeostatic (wound healing) situation. In the subsequent state of graft healing, K19 expression became strictly restricted to keratinocytes in the stratum basale, before it was completely down regulated at the establishment of skin homeostasis. Interestingly, we also found K19 expressing keratinocytes restricted to the stratum basale in human skin of very young children (not older

than 1.5 years). In children older than two years or adults K19 expression was no longer detected. We speculate that K19 is expressed in keratinocytes whenever rapid expansion (through massive proliferation) of skin has to occur (Pontiggia et al. 2009). The here observed induction of K19 upon UVB irradiation suggests a reactivation of exactly this mechanism, and is in line with the notion that a wound healing process was induced upon UVB irradiation.

UVB irradiation induces proliferation of both basal and suprabasal epidermal keratinocytes

A large body of evidence gained in adult mice suggests that constant keratinocyte proliferation occurs in the basal layer of the epidermis. Suprabasal proliferation in murine skin was only described during a short period in early embryogenesis (Kulukian and Fuchs 2013).

In contrast, our observations reveal that in homeostatic MelSkin (and normal human skin) about 70% of the Ki67 positive keratinocytes are located in the first suprabasal epidermal layers. In human skin, basal keratinocytes differ significantly from suprabasal keratinocytes, which is underlined by the distinct expression of markers, such as K15 (exclusively in the basal layer) and K10 (exclusively in suprabasal and upper layers). In accordance with our above-mentioned observation there is now increasing evidence indicating that in human skin Ki67-positive keratinocytes are mainly located in the first suprabasal layers (Lee et al. 2002; Kiowski et al. 2012), though suprabasal proliferation was never specifically noticed. We also observed this phenomenon when we systematically analysed human IFE and skin substitutes of different stages (manuscript in preparation). Furthermore, there is a recent publication by Nöske et al., who identified suprabasal proliferation as a common condition in human interfollicular epidermis. The authors screened

human skin samples from different parts of the body and found up to 70% of Ki67-positive keratinocytes in the first suprabasal layers (Nöske et al. 2016).

Previously published data revealed that UVB exposure induced keratinocyte proliferation in human skin with a peak between 2 and 5 days (van der Vleuten et al. 1996). This finding is confirmed by our data. Moreover, we found an initial peak of proliferation in the basal layer, which was unexpected, given the mostly suprabasal proliferation in homeostatic epidermis. Interestingly this proliferation of epidermal keratinocytes goes along with the "multilayering" of the stratum basale. At least 3 layers of basal keratinocytes were K15 positive, 1 week after UVB exposure. As basal keratinocytes are the first ones to proliferate after UVB irradiation, it is tempting to speculate that the two additional basal keratinocyte layers are derived from the initial singular layer of K15 positive cells (stratum basale). Upon irradiation a huge number of usually quiescent (label retaining) basal keratinocytes may be pushed into an "emergency activation", hence be forced to withdraw from cell-cycle G0-phase and undergo mitosis. Given the recent observation that in homeostatic human skin it is predominantly the suprabasal keratinocyte fraction that proliferates, one may speculate that there are two distinct fractions of proliferating keratinocytes. One suprabasal fraction, proliferating during homeostasis, thereby constantly renewing the epidermis, and a fraction of quiescent basal keratinocytes that can be induced to proliferate in "emergency situations" such as wounding and irradiation. Notably, this model fits ideally with the model suggested by the group of Clevers, which implies that both quiescent (out of cell cycle and in a lower metabolic state) and active (in cell cycle and not able to retain DNA labels) stem cell subpopulations may coexist in several tissues, in separate yet adjacent locations. These authors propose that quiescent and active stem cell populations have separate but cooperative functional roles (Li and Clevers 2010). In line with Li et al. we find that the proliferative activation of usually quiescent basal keratinocytes goes along with

the reduction of Wnt inhibitor expression, hence most likely with the activation of Wnt signaling. This however, remains to be demonstrated by subsequent experimentation.

Proliferation of basal keratinocytes correlates with the suppression of Wnt inhibitors in keratinocytes upon UVB exposure

The interplay between Wnt ligands and Wnt inhibitors was found to regulate proliferation of stem cells in intestinal crypts and hematopoetic, neural and liver stem cells (Barker et al. 2010; Kalani et al. 2008; Li & Clevers 2010). Moreover, Wnt signaling plays an important role in hair follicle proliferation and differentiation (Steingrímsson et al. 2005), in cutaneous wound repair (Fathke et al. 2006), and in tumor formation (Arwert et al. 2012). The role of DKK3 in normal and pathological skin is only partially understood. DKK3 was reported to be downregulated in hyperproliferative skin (Du et al. 2011). In contrast, DKK3 upregulation was described in mouse and human hair follicle bulge regions. It was proposed as a marker for hair follicle stem cells (Ohyama et al. 2006; Tiede et al. 2007). Lim et al. showed the strong expression of DKK3 protein in all suprabasal cells in the murine epidermis and also in some single basal layer cells. These authors suggested that DKK3 protein accumulates at high levels in the suprabasal layers thereby preventing suprabasal cells from proliferation (Lim et al. 2013). Although this was shown in murine skin, the same mechanism may be present in human skin.

Importantly, and in contrast to murine skin, we show here the typical expression pattern of DKK3 in the homeostatic human epidermis, and here not only in suprabasal, but also in most basal keratinocytes. The observed reduction of DKK3 expression in the vast majority of basal keratinocytes, one week after UVB irradiation, and the reappearance of basal DKK3 expression in MelSkin 5 weeks after UVB exposure, correlates with the proliferation dynamics observed in epidermal keratinocytes.

In line with these findings, and as previously reported (Schlüter et al. 2013), we observed WIF1 expression in differentiated suprabasal keratinocytes as well as in non-proliferating basal cells in homeostatic epidermis. The loss of WIF1 expression in proliferating keratinocytes, both in culture and *in vivo*, is in accordance with Schlüter et al., who suggested that WIF1 negatively regulates keratinocyte proliferation and is required to keep stem cells quiescent and provides differentiated cells from proliferation (Schlüter et al. 2013). Downregulation of WIF1 was also found in psoriatic skin, exhibiting persistently high proliferation rates (Gudjonsson et al. 2010).

WIF1 expression persists in melanocytes after UVB exposure

It was previously reported that WIF1 is expressed in melanocytes *in vitro,* when these were grown on cell culture plastic and *in vivo* respectively. Furthermore, WIF1 expression is thought to promote melanogenesis (Park et al. 2014). We found that melanocytes continued to express WIF1 after UVB exposure. Accordingly, and in contrast to a certain fraction of epidermal keratinocytes, these melanocytes were not stimulated to proliferate upon UVB irradiation.

In conclusion, when MelSkin, engrafted on immunecompromised rats, is irradiated by UVB, it exhibits a physiological pattern of reactions, consisting of tanning, a massive transient proliferation of epidermal keratinocytes, and a significant temporary wound healing-like response.

The observed UVB-induced proliferation of keratinocytes goes along with the reduced expression of Wnt inhibitors. In contrast, melanocytes are not triggered to proliferate by UVB exposure, yet they are stimulated to produce, transport and transfer melanin to epidermal keratinocytes. This report also hints at the existence of two distinct fractions of proliferating keratinocytes in human skin: a) suprabasal keratinocytes maintaining the integrity of the

epidermis during skin homeostasis, and b) quiescent basal keratinocytes that can be induced to proliferate by “emergency situations” such as wounding and irradiation.

Finally, MelSkin represents a new generation of pigmented bio-engineered skin substitutes for clinical application, but also provides a valuable model for experimental and screening purposes.

Conflict of Interest

The authors state no conflict of interest.

Acknowledgments

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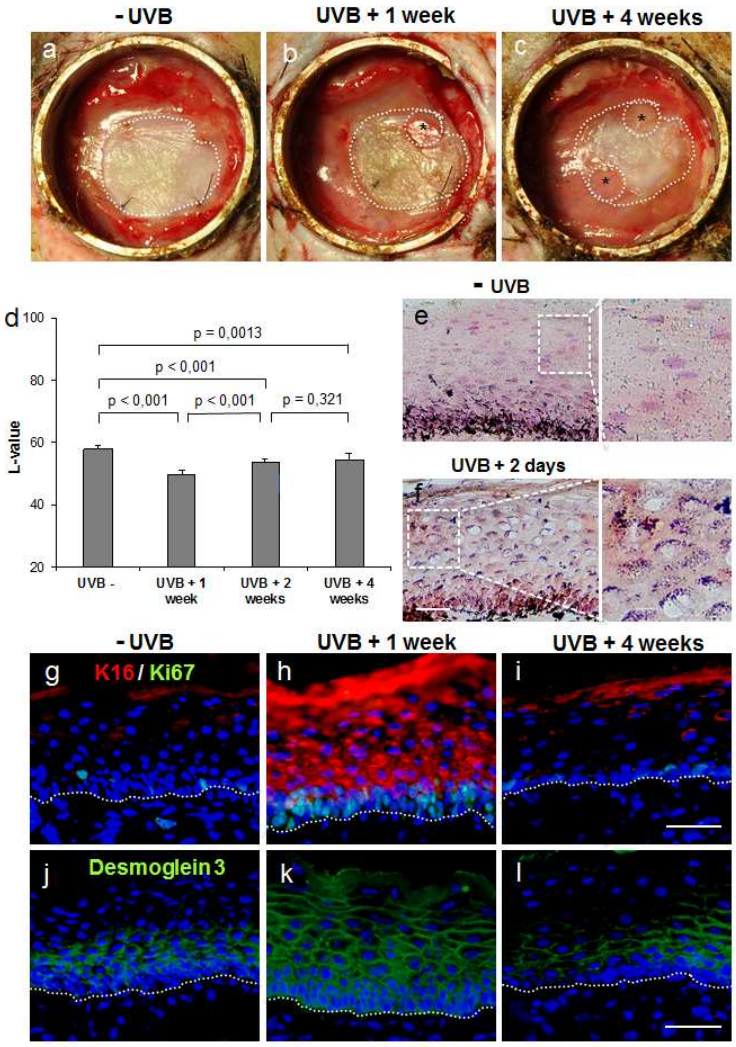

Figure 1: Tanning and wound induction in MelSkin after UVB irradiation

(**a-c**) Skin substitute 4 weeks after transplantation before UVB irradiation (**a**), 1 week (**b**) and 4 weeks (**c**) after irradiation. Taken punch biopsies (*) decreased surface (**b, c**). Tanning 1 week after irradiation (**b**), and confirmation by chromameter measurement (**d**): The L-value significantly decreased 1 week after irradiation representing a darker skin color, and reincreased after 2 and 4 weeks. (**e,f**) Fontana-Masson staining of MelSkin before (**e**) and 2 days after irradiation (**f**) displaying supranuclear melanin caps in keratinocytes extending to upper suprabasal layers. Stainings for Keratin 16 (red), Ki67 (green) (**g-i**), and Desmoglein 3 (green) (**j-l**) show induced marker expression 1 week after irradiation (**h,k**). White dotted line: dermo-epidermal border. Scale bars: 50µm

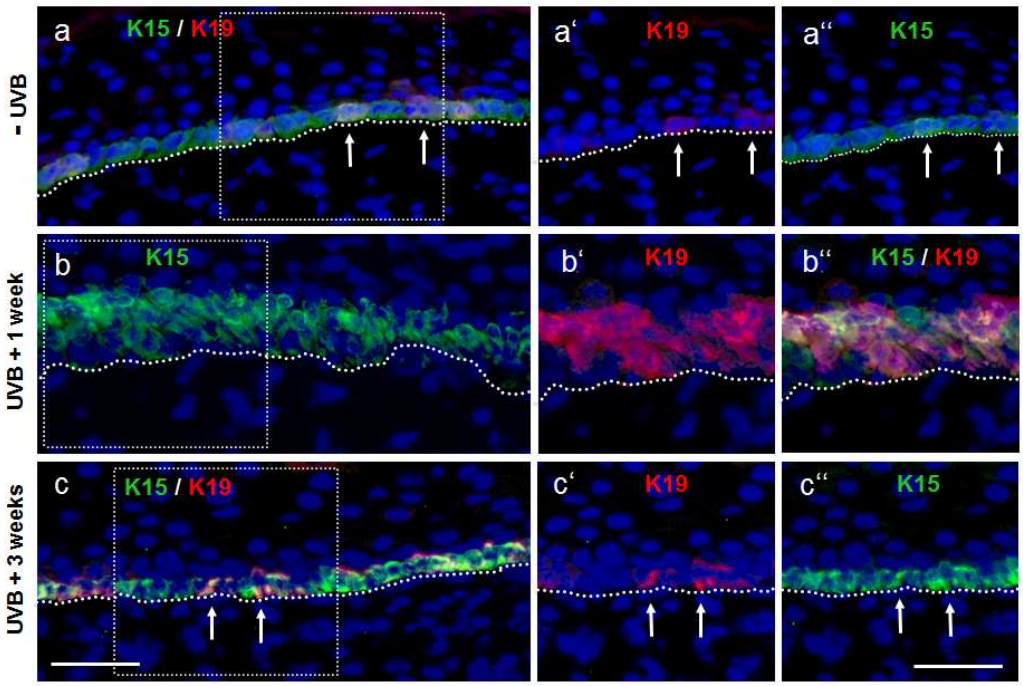

Figure 2: Induction of Keratin 19+/ Keratin 15+ basal keratinocytes after UVB exposure

(**a, b'', c**) K19(red)/K15(green) staining of MelSkin. **(a, a', a'')** During normal skin homeostasis, before UVB irradiation, few single keratinocytes in the basal layer show a weak K19 expression (white arrows). K15 is expressed homogeneously in the entire basal monolayer. (**b, b', b''**) 1 week after irradiation, "multilayering" of the stratum basale and a massive increase in K19/K15 double positive cells can be observed. (**c, c', c''**) 3 weeks after irradiation the initial singular layer of K15 positive cells is restored and only few basal cells are still $K19^+$ (white arrows). White dotted line: dermo-epidermal border. Scale bar: 50µm

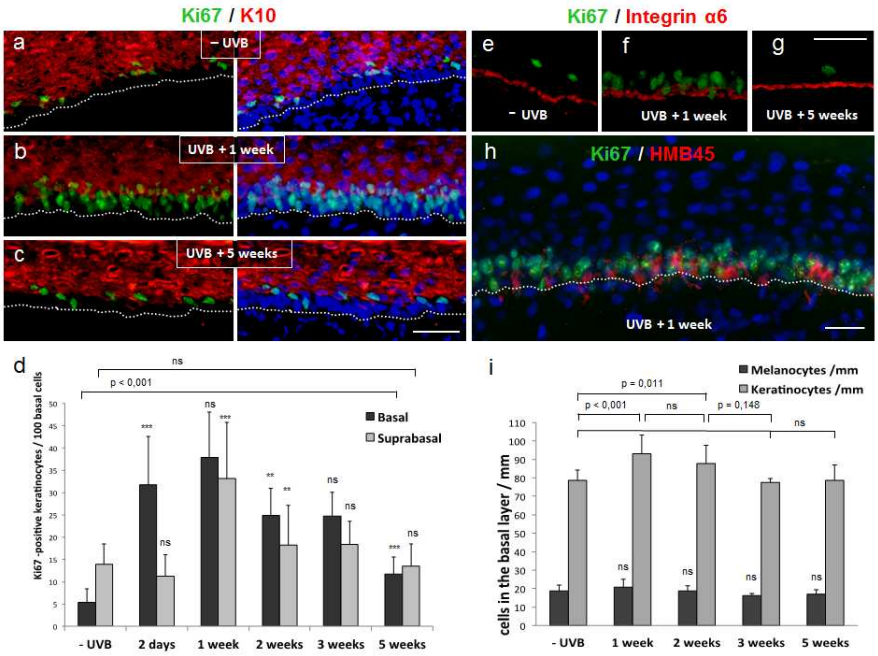

Figure 3: Organized proliferation of basal layer keratinocytes in the epidermis

(**a-c**) Staining of Ki67 (green) before (**a**), 1 week (**b**) and 5 weeks (**c**) after UVB exposure. Keratin 10 (red) is only expressed in suprabasal layers. (**d**) An initial increase of basal Ki67^{+} cells 2 days after irradiation, followed by a peak of proliferation in both basal and suprabasal compartment after 1 week. Proliferation decreased, reaching its original level 5 weeks after irradiation. (**e-g**) Staining of Ki67 and basement membrane marker Integrin alpha 6 reaffirms the massive increase of basal cell proliferation. Ki67/HMB45 staining (**h**) shows a constant number of melanocytes in the basal layer after irradiation (**i**), revealing no evidence for melanocyte proliferation after irradiation. White dotted line: dermo-epidermal border. Scale bars: 50µm

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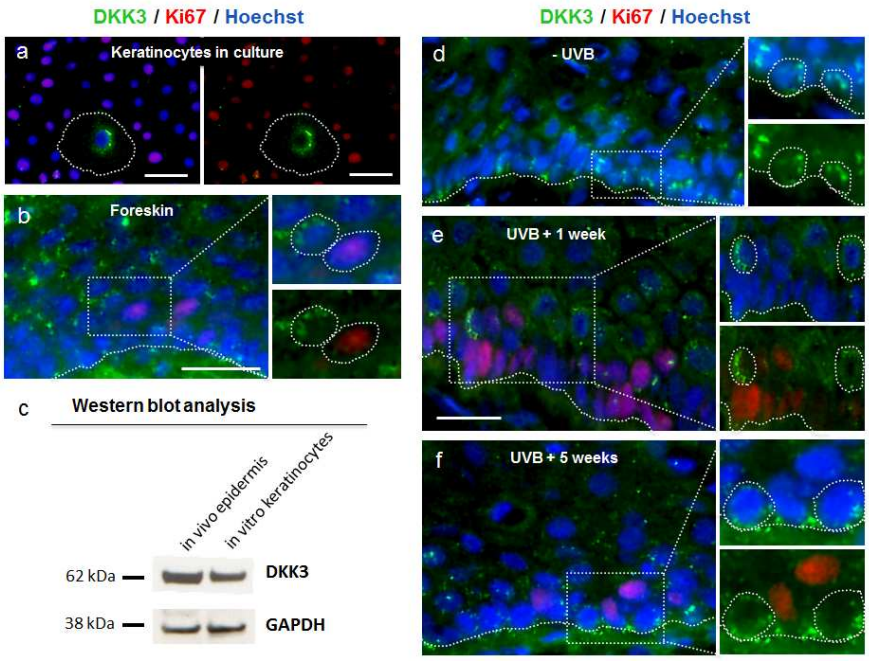

Figure 4: Absence of DKK3 expression in proliferating basal layer keratinocytes

(**a, b**) Staining of Wnt inhibitor DKK3 (green) and Ki67 of keratinocytes in culture and human foreskin shows the typical expression pattern of DKK3 with spotted vesicles around the nucleus. In culture, only few single keratinocytes are $DKK3^{+}$ and notably $Ki67^{-}$. (**c**) Western blot analysis reveals decreased levels of DKK3 protein in proliferating keratinocytes in culture (*in vitro*), compared to foreskin keratinocytes (*in vivo*). (**d-f**) In MelSkin, DKK3 expression seen in basal and lower suprabasal layers before UVB exposure (**d**) and 5 weeks (**f**) after exposure. 1 week after irradiation (**e**), DKK3 expression is lost in the basal layer of MelSkin, especially in $Ki67^{+}$ cells (red). White dotted line: dermo-epidermal border. Scale bar: 25µm

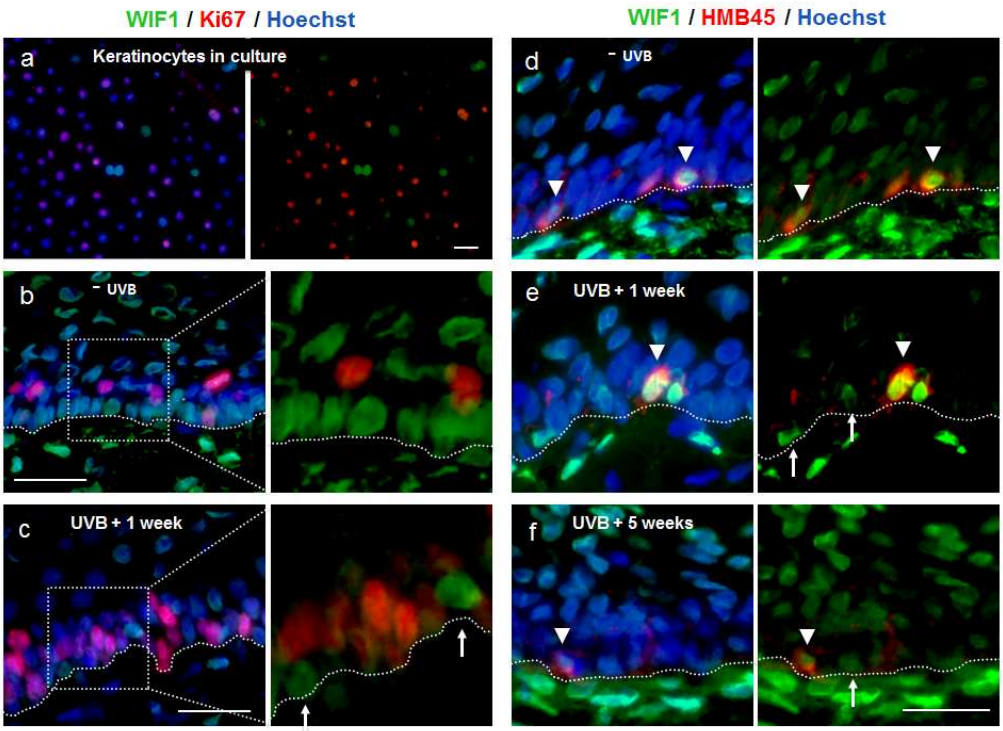

Figure 5: WIF1 is expressed in melanocytes, but is lost in proliferating basal layer keratinocytes and in culture

(**a**) WIF1/Ki67 staining of keratinocytes in culture: Cells are either Ki67 (red) or WIF1 (green) positive. (**b**) WIF1 is expressed in most suprabasal and basal cells of MelSkin epidermis and dermal fibroblasts. (**c**) Many basal layer keratinocytes lost WIF1 expression 1 week after UVB irradiation. Some single basal cells still express WIF1 and are notably $Ki67^{-}$ (white arrows in **c**). (**d-f**) $HMB45^{+}$ melanocytes (red) emerge with a more intense expression of WIF1 (green), maintained after irradiation (white arrowheads). However, not only melanocytes remain $WIF1^{+}$, also a few basal keratinocytes continue to express WIF1 (white arrows in **e, f**). White dotted line: dermo-epidermal border. Scale bar: 25µm